BROAD-SPECTRUM BIOSENSOR FOR MONITORING CURRENT AND FUTURE TRANSGENIC PLANT TECHNOLOGIES FOR INSECT CONTROL

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Abstract

An alternative to immunochemical and molecular assays was developed to detect insect resistant traits found in transgenic cotton. The biosensor used was susceptible neonates of the tobacco budworm, *Heliothis virescens*. The assay was a feeding disruption test originally developed for monitoring insect resistance. From a few drops of sample extract, the assay was able to detect the presence or absence of the trait. Proof of concept was demonstrated for Bollgard II and WideStrike. Among the advantages of this assay approach are low cost, long shelf life at room temperature, minimal biochemical training needed, and the use of a single assay kit to detect any of the existing insect resistant traits found in transgenic cotton. No additional development should be needed for new traits that may become available in the future including potential RNAi approaches to insect control. Another advantage is that the assay only detects intact, functional toxin; protein or nucleic acid degradation products that have lost their activity but could potentially be detected by immunoassay or PCR would be excluded. The development of antibodies, the need for expensive enzymes, purified toxin or other analytical chemicals, and the need for multiple kits for each trait potentially present in the different and expanding number of commercial transgenic plant varieties is eliminated.

Introduction

The application of insect-resistant transgenic crops is expanding at double-digit rates (Christou et al., 2006), and it appears this technology will remain as an important part of agricultural production in the foreseeable future. The insect toxins most often used in transgenic plants are derived from the bacterium, *Bacillus thuringiensis* (Bt). All existing evidence indicates that these proteins are safe to the environment, wildlife and human health. Also, new technologies are rapidly being developed for alternative proteins to the current Bt Cry toxins as well as approaches using double stranded RNA (dsRNA).

There are many important benefits to the use of insect-resistant transgenic crops which include improved insect control, lower use of synthetic chemical insecticides and their reduced negative effects on non-target organisms, and the simplification of pest management which allows farmers to concentrate on other aspects of crop production. However, there is also a concern by the public, government regulatory agencies and the scientific community about the release of transgenic organisms and products derived from these systems into the environment and their intentional and unintentional distribution. Today, we live in a global economy which greatly impacts agriculture. For example, the international seed trade has increased from 860 million US dollars in 1970 to a multi-billion dollar industry today. The potential for the movement of transgenic plants is significant. Genetically enhanced foods, although proven safe, are not yet accepted by many in the general public both in the US and abroad. At the same time, there is an increasing interest in natural foods and organic farming at the exclusion of engineered plants. It is well recognized that diagnostics will be needed to monitor for transgenic crops and products from these plants in our environment and commerce.

The current diagnostic technologies used in agriculture for the detection of insect-resistant plants can be divided into two basic categories: (a) immunochemical assays (enzyme-linked immunosorbent assays (ELISAs) and lateral flow strip tests) for the detection of protein toxins and (b) molecular assays using polymerase chain reaction (PCR) and nucleic acid hybridization for the detection of the genetic material (DNA and RNA) that produce the protein toxin in the plant. The strip test has many features that are well-suited to agricultural diagnostics, for example, its ease of operation and short assay time. Disadvantages include the need to develop antibodies which require the use of animals, the cost of antibody production which includes access to purified protein toxin from the different plant

varieties in commercial production, and high antibody specificity on one hand and cross-reactivity on the other. In respect to high specificity, in some cases it may be necessary to have a separate antibody for each potential protein toxin that might be produced. Immunochemical assays and especially monoclonal antibodies because of their specificity are capable of detecting a single site within the protein toxin. This site could be present in a sample where the intact protein has been degraded to the point of being non-functional. Antibody-antigen assays also suffer from unpredicted, non-specific interactions with plant material or other components in the sample to be assayed. Furthermore, immunochemical assays will not work for next generation control strategies like plant-derived RNAi. ELISAs have the advantages over lateral flow strip tests of being quantitative and allowing multiple simultaneous assays. However, the assays can require longer assay times (2-8 hours), expensive laboratory equipment, and personnel highly trained in biochemistry. They also have many of the same limitations found in the strip test due to their dependency on the antibody-antigen interaction. Antibodies in general, are labile which reduces the assay shelf life and requires specialized storage conditions like refrigeration.

There is a growing interest in nucleic acid based assays because of advances in a variety of technologies such as quantitative PCR, isothermal amplification, digital gene expression, hybridization and microarrays. Nucleic acid detection methods most often require gene or mRNA amplification, expensive reagents and laboratory equipment, highly trained technicians, and can be time consuming. There have been significant improvements in making the assays quantitative but again the technology for this is expensive. Like immunoassays, nucleic acid detection can be highly specific although it is possible to multiplex PCR primers to examine multiple targets simultaneously. PCR is highly sensitive, in theory able to detect a single nucleic acid molecule. The detection of a nucleic acid that codes for an insect toxin does not necessarily indicate that the toxin is present and active; this is an important negative aspect for this approach. Also, with the rapid expanding number of protein toxins being developed by different companies, the development of stacked genes and RNAi approaches for pest control, and the need for a separate immuno- or molecular-assay for each protein or dsRNA in these plants using the current detection technologies, it seems reasonable that alternative strategies should be investigated.

We have been working for some years in improving bioassay approaches for the detection of insect resistance to pesticides and have developed what is commonly referenced as feeding disruption tests (FDTs) for monitoring resistance to larval and adult insects. The assays have been developed for a variety of compounds including chemical, protein and nucleic acid insecticides and for a variety of larval and adult insect pests which include but are not limited to the tobacco budworm, cotton bollworm, cabbage looper, diamond back moth, beet armyworm, Colorado potato beetle, plant bugs, and stink bugs (Bailey et al. 1998, 2000, 2001; Roe et al. 2000, 2002, 2003, 2004, 2005; Khalil et al. 2002; van Kretschmar et al. 2009, 2011 (in press); Cabrera et al., 2010). The kit consists of a specially designed white plastic 16-well plate with recessed, hydrateable meal pads which are mass-produced robotically. The meal pads contain a diagnostic dose of insecticide and a blue indicator dye to monitor insect feeding. The appearance of blue feces (a measure of feeding rate) can easily be seen on the background of the white plate after a 4-24 h incubation period in resistant insects. The absence of blue feces occurs because the susceptible insects are intoxicated by the insecticide and do not feed. Extensive probit modeling using Cry1Ac toxin was conducted to validate this technology for multiple field strains of the budworm and bollworm from the SE US (Bailey et al. 2001). These kits also have been field validated for different chemical insecticides. FD tests have been developed for spinosad, permethrin, indoxacarb and many other chemical insecticides. The advantage of the larval FDT kit is that it provides a rapid diagnosis of resistance using a standardized format that is easily adaptable to different applications, provides a method to economically mass produce the kits, provides an off the shelf ready to use assay (just add a drop of water), can be used for multiple species, and assays for multiple mechanisms of resistance simultaneously. An additional advantage the hydrateable meal pads provide is an easy method for the incorporation of test sample material into artificial diet which then can be used to conduct an insect bioassay. We have shown before (Cabrera et al., 2010) that leaf extract from cotton plants can easily be incorporated into these hydrateable meal pads and used as a method for monitoring insect resistance to plants with stacked genes like Bollgard II. The current paper is the first report of the use of these FDTs as a method for the detection of transgenic plant material and an alternative to immunochemical and molecular assays for transgenic cotton traits used for insect control.

Materials and Methods

Cotton Plants and Leaf Extract Preparation

Leaves from 8-week old cotton plants were used to prepare leaf extracts. Cotton varieties used were (a) non-Bt cotton PHY 425 RF (Dow AgroSciences, Indianapolis, IN), (b) Bollgard II cotton DP 161 B2RF expressing Cry1Ac and Cry2Ab toxins (Monsanto, St. Louis, MO), and (c) WideStrike cotton PHY 485 WRF expressing Cry1Ac and Cry1F (Dow AgroSciences, Indianapolis, IN). The plants were grown under greenhouse conditions with the temperature ranging from 22-31ºC and under natural light. After harvest, the leaves were stored at -80ºC until used. A leaf extract stock solution was prepared for each cotton variety using 4 ml of distilled water/gram leaf tissue or 0.25 µg leaf/µl. The leaf tissue and water were homogenized for 5 min using a Polytron (PCU, Kinematica, Switzerland) and then filtered through glass wool. The eluant was stored at -80ºC until needed. Concentration is expressed in this paper in units of µg leaf/µl or µg leaf/meal pad.

Insects

To determine a dose for Bollgard II and Widestrike cotton that would prevent fecal production, we used neonate (0-24 h old) tobacco budworms (*Heliothis virescens*) from the Bt-susceptible strain Hv02, reared under laboratory conditions on artificial diet (Burton, 1970) at the insectary facility of North Carolina State University (Raleigh, NC, USA).

Bioassay Description

The bioassay is based on a previous technique described by Roe et al. (2005). Assays were conducted in 16 well FDT plates essentially as described by van Kretschmar et al. (in press). In the current study, each well of the plate was designed to contain 200 µl of a hydrateable heliothine artificial diet (Burton, 1970) prepared as described by van Kretschmar et al. (in press). This diet was rehydrated for the studies that follow with 170 µl of leaf eluant and incubated at room temperature in open air for 2 h before use. The bioassay was then initiated by the addition of a single 0-24 h old (from egg hatch) neonate of the tobacco budworm to each well. The wells of the plates were then sealed with an adhesive cover and incubated for 24 h at 27^{o}C with a 14:10 LD cycle. After this incubation, the number of blue fecal pellets produced by each insect was determined by observation using a dissecting scope. The blue feces are effortlessly seen on the white background of the plastic well.

Estimation of Diagnostic Dose

The bioassay described in the previous section was utilized to determine a diagnostic dose for Bollgard II and WideStrike eluant which would prevent the production of blue feces for susceptible insects (the biosensor). These studies were conducted with 0.7, 1.3, 2.6 and 5.3 µg of Bollgard II cotton per meal pad and 5.3, 10.6, 21.2 and 42.5 µg of WideStrike cotton per meal pad using the eluants described earlier. The control in these experiments was non-Bt cotton at the rate of 1.3 and 42.5 µg per meal pad for Bollgard and WideStike, respectively. For each Bollgard II and WideStrike dose tested, four 16-well plates were used (a total of 64 larvae). The diagnostic dose was determined as the concentration of leaf material that resulted in 0-2 fecal pellets per larva after 24 h.

Statistics

Comparisons between each cotton variety (Bollgard II and WideStrike) and their corresponding non-Bt control were conducted with t-tests for validation of the dose response and diagnostic dose. The variation plotted was $\pm$ 1 standard error of the mean.

Results and Discussion

In the assays conducted, two different sources of transgenic material with traits for insect resistance were examined, Bollgard II and WideStrike. As shown in Figs. 1-2, extract from these plants reduced fecal production significantly even at the lowest dose tested as compared to conventional cotton plants. The assumption was made that this reduction in fecal production was the result of the effects of the expressed Bt Cry toxins in Bollgard II and WideStrike on neonate feeding in the tobacco budworm. This assumption seems to be reasonable since Cabrera et al (2010) reported that neonates from two different Bt resistant strains of the tobacco budworm (YHD2 and CxC) were

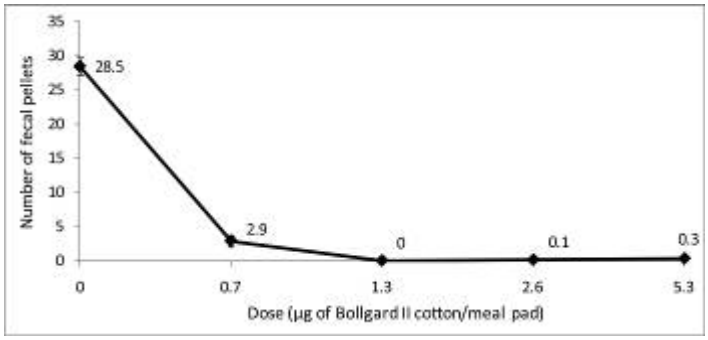

Figure 1. Effect of dose of Bollgard II cotton leaf material per meal pad on the number of blue fecal pellets produced per neonate of the tobacco budworm after a 24 h incubation. The zero dose was conventional cotton. The description for the preparation of leaf homogenate is described in the Materials and Methods. Each point plotted is ± 1 standard error of the mean which in some cases did not exceed the size of the symbol.

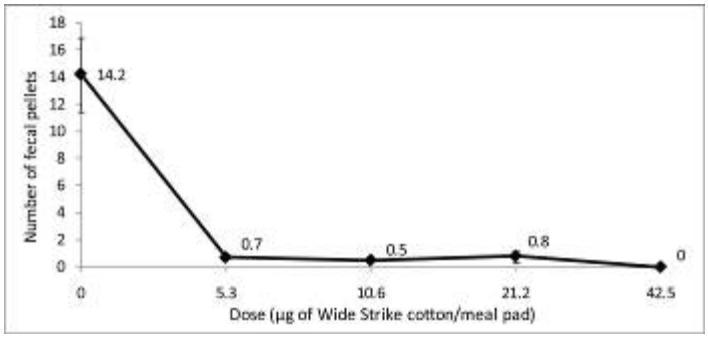

Figure 2. Effect of dose of WideStrike cotton leaf material per meal pad on the number of blue fecal pellets produced per neonate of the tobacco budworm after a 24 h incubation. The zero dose was conventional cotton. The description for the preparation of leaf homogenate is described in the Materials and Methods. Each point plotted is ± 1 standard error of the mean which in some cases did not exceed the size of the symbol.

able to successfully produce blue feces on high concentrations of Bollgard II extract in meal pads. No other independent studies were conducted in the current study to determine the presence of the Cry toxins in the Bollgard II and WideStrike leaf extracts.

Figs. 1-2, show a dose response between the concentration of Bollgard II and WideStrike leaf material in the meal pads and blue fecal production. As the concentration of the leaf homogenate increased, fecal production decreased. At the highest concentration tested, the average fecal production approached zero. These results are proof of concept that budworm neonates can be used to detect in a dose dependent relationship, the presence of Cry toxins in plant samples. van Kretschmar et al. (in press) showed previously a similar dose response in neonates of both tobacco budworms and cotton bollworms (*Helicoverpa zea*) for purified Cry1Ac, Cry1F, and Cry1Ab toxin. Fig. 3 shows a typical all or none FD test result for Bollgard II where (A) is conventional cotton and (B) is Bollgard II cotton leaf extract at the highest dose tested (Fig. 1) and (C) another conventional cotton control conducted simultaneously with (D) the WideStrike leaf extract at the highest dose tested (Fig. 2). The absence of fecal production in (B) and (D) is an indication of the presence of the plant trait for insect resistance.

This FD test for insect resistant cotton traits has several advantages over that of immunoassay or molecular assays: (a) the assay should be less expensive since it consists of only a small hydrateable insect meal per assay well in a 16-well plate format, (b) the assay should have an extended shelf life at room temperature since no antibodies, enzymes or other reagents are needed to conduct the assay, (c) the assay does not require laboratory equipment or personnel highly trained in biochemistry or molecular biology, (d) a single assay format can be used to test any engineered insect toxin eliminating the need for multiple kits to measure each toxin separately, (e) the assay format is

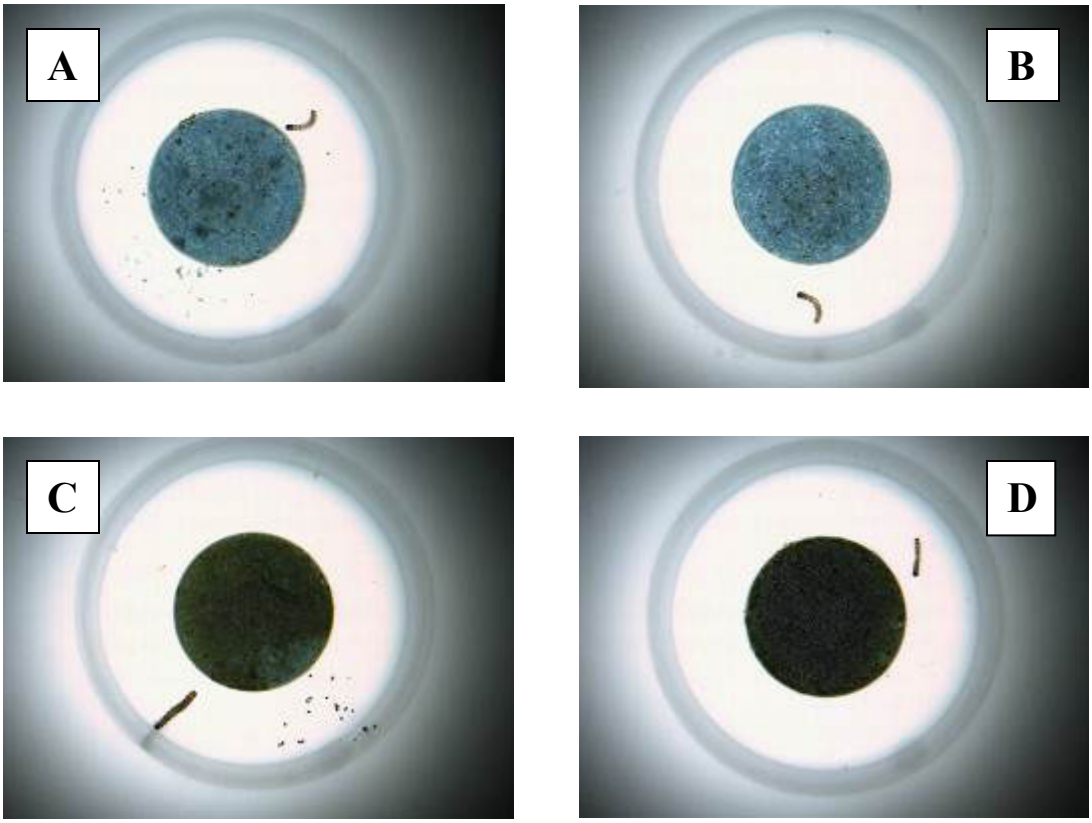

Figure 3. Typical assay response for (A) the control (conventional cotton with no-Bt toxins) with (B) its corresponding Bollgard II treatment at the highest concentration tested (Fig. 1); and typical assay response for (C) the control (conventional cotton with no-Bt toxins) with (D) its corresponding WideStrike treatment at the highest concentration tested (Fig. 2). The center dark circle is the meal pad hydrated with leaf homogenate surrounded by the white background which is the bottom of each well in the 16-well assay plate (the plastic cover was removed to obtain the photograph). The small dark dots in (A) and (C) are blue fecal pellets produced by neonates of the tobacco budworm after a 24 h incubation with the meal pads. See Materials and Methods for more details.

immediately usable to detect new commercial cotton varieties and insect resistant traits with no further assay development needed, (f) the assay is also adaptable to next generation control systems like RNAi, (g) the detection system is for active toxin only (there are currently no assays available like ELISA or nucleic acid tests that can detect or measure the insecticidal activity), (h) the same assays can be used as artificial cotton leaves to detect the evolution of insect resistance to transgenic cotton (Cabrera et al., 2010), and (i) the assays do not require experience in biochemistry or molecular biology. Leaf extract preparation can also be made simple since the assay format is a bioassay; no special sample clean up is needed, and the method is not subject to interfering compounds in the sample like that often reported for immunochemical or molecular assays.

For fresh leaf material, the sensitivity of the new FD test described here well exceeds the concentration of toxin in the leaf. However, for materials derived from cotton or samples that have been stored for extended periods or processed, this may not be the case. Additional studies will be needed to better define the minimum detection limits. Preliminary studies also suggest that choice studies between conventional and plants expressing Cry toxins can be used to increase sensitivity and/or reduce assay time to as little as 30 min (Roe, van Kretschmar and Ezzeldin, unpublished). One disadvantage of the current FDT for insect resistant traits is that the method requires access to eggs or neonates of the tobacco budworm or another moth pest that is susceptible to the toxin being expressed. In the case of eggs, they can be stored for several days at 4^{o} C before allowing the eggs to hatch. These insects are also commercially available and in culture in many industry and university labs.

Summary

The current studies show that the artificial leaf FDT assay for monitoring insect resistance to pyramided cotton like Bollgard II as described by Cabrera et al. (2010) can also be used as a method for monitoring unknown samples containing Bt Cry toxins for Bollgard II and WideStrike and should be applicable to any insecticidal toxins that affect the insect test species, in this example, *H. virescens*. The assay simply involves applying an aqueous sample extract to a hydrateable meal pad followed by the addition of a neonate. After a 24-h incubation, the absence of blue feces is an indication of the presence of a functional toxin. The detailed advantages of this assay over that of immunochemical and molecular assays for cotton traits for insect resistance are discussed.

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